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STRANGE KINETICS of single molecules in living cells

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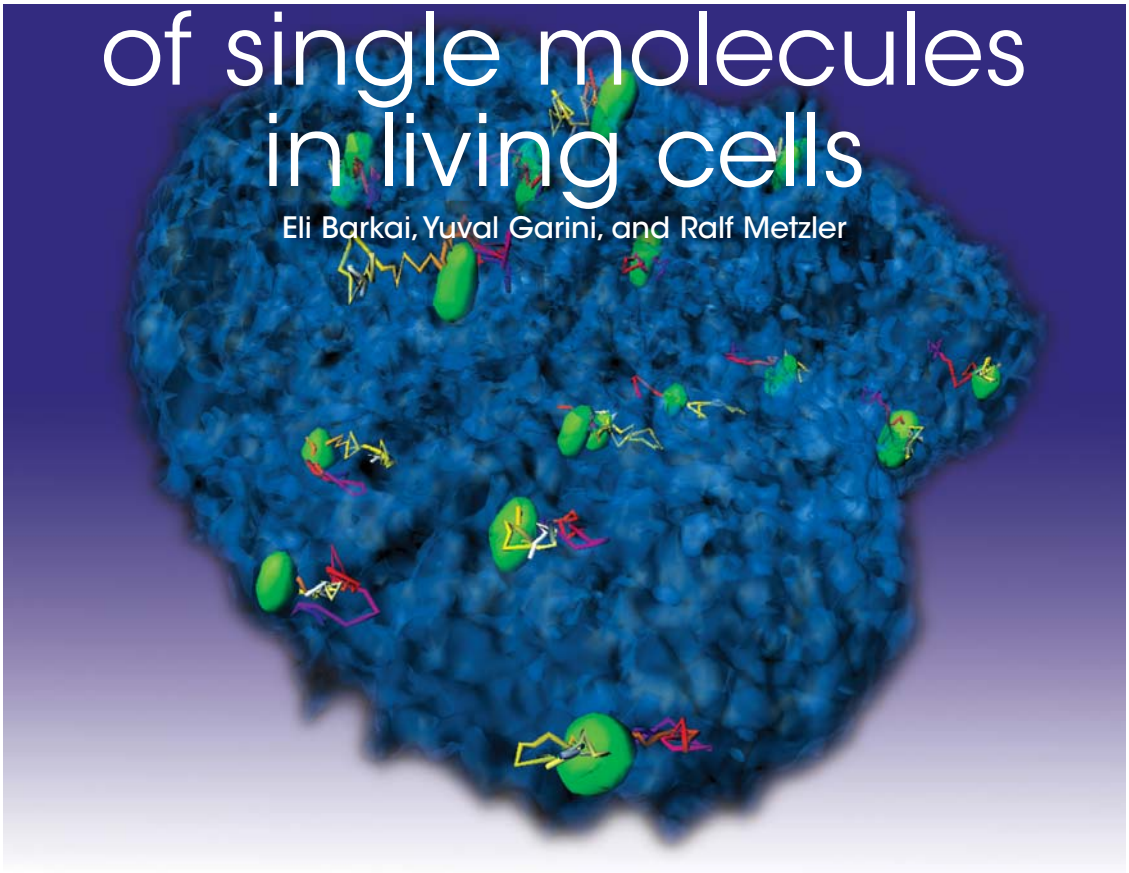

The irreproducibility of time-averaged observables in living cells poses fundamental questions for statistical mechanics and reshapes our views on cell biology.

For centuries, physical imaging tools have been opening new frontiers in biology. The discovery of the cell nucleus by Scottish botanist Robert Brown was made possible by early-19th-century light microscopes, and DNA was unveiled by mid-20th-century x-ray diffraction imaging.

During his observations in the 1820s, Brown made another discovery, which has come to bear his name. He was startled to see the jittering, lifelike motion of small particles enclosed in pollen grains. He used control experiments with dust particles to rule out the notion that the movers had to be living "animalcules." In the early 20th century, Brownian motion became the subject of theoretical investigations by Albert Einstein, Paul Langevin, Marian Smoluchowski, and others.

Following single molecules

Now once again, another connection between biology and physics is being forged, this time by a new imaging technique called single-molecule spectroscopy.[1] Tracking individual molecules or small tracer particles in living cells yields insight into the molecular pathways that underlie cellular regulation, signaling, and gene expression. Researchers may soon be able to follow the trajectory of an individual messenger RNA molecule from its production—by the transcription of a sequence encoded in a specific gene on the cell's DNA—to its conversion into a protein by a ribosome. Although some individual proteins are too small to follow by single-molecule tracking, certain proteins that occur in extremely low concentrations could be followed by molecular buoys that emit light when the proteins temporarily dock at them.

The light emitted from a single molecule moving through a living cell is just one example of dynamics in complex animate or inanimate systems in which one encounters complicated time variation of observables. Usually there's little hope of determining those variations in detail, except for some averaged features. Such averages are usually taken over suitable ensembles: One observes many molecules and averages the results. But in single-molecule experiments, one observes the same particle for a long

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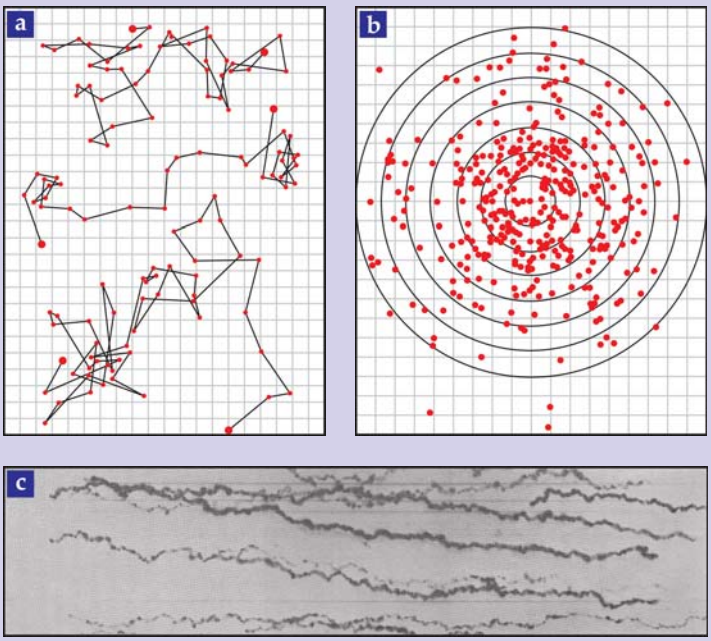


Figure 1. Analyzing Brownian motion by different approaches. **(a)** In 1908 Jean Perrin recorded individual trajectories of small putty particles in water at 30-second intervals (red dots). **(b)** He then plotted all the 30-second displacements, shifted to a common origin, and obtained an ensemble diffusion constant by fitting a Gaussian to the distribution of points. (Adapted from ref. 15.) **(c)** Six years later, Ivar Nordlund traced, on moving film strips, individual trajectories of mercury particles in water as they slowly settled to the bottom. The waviness of the curves is due to Brownian motion. He analyzed the trajectories to obtain time-averaged mean squared displacements. (Adapted from ref. 16.)

time, and the reported quantities are then time averages rather than ensemble averages.

Statistical physics usually deals with so-called ergodic systems, for which time and ensemble averages are the same. That equality is codified in the ergodic theorem at the heart of statistical mechanics. But when tracking chemically identical molecules diffusing in *living* cells, one routinely finds that the time averages vary from one molecule to the next. Such apparent randomness of time averages is in complete contrast to our experience of the Brownian motion of molecules in the dilute conditions of a test tube.

In that sense, single-molecule tracking is shifting our point of view away from the usual ergodic line of thought. One can no longer safely assume that measurement of one molecule's motion yields the dynamical behavior of another identical molecule under the same physical conditions. This article seeks to provide an overview of the current experimental state of single-molecule tracking in living cells and of how statistical physicists are developing new tools to interpret those measurements. In particular, we focus on the observation of distinctly nonergodic behavior and large deviations from Brownian motion. We will also discuss some potential implications of that "strange kinetics" for cell biology.

Brownian motion

Three years after Einstein's historic 1905 paper on Brownian motion, Jean Perrin in Paris introduced systematic single-particle tracking. Because the Brownian trajectories were relatively short, he used ensemble averages over many particle traces to obtain meaningful statistics. A few years later, Ivar Nordlund in Uppsala, Sweden, conceived a method for recording much longer time series. That let him determine time averages over individual trajectories and thus avoid averages over ensembles of particles that were probably not identical (see figure 1).

To understand how the approaches of Perrin and Nordlund are connected to each other, imagine dripping a drop of ink into water. The initially localized blob will spread according to the laws of diffusion such that its mean squared displacement (MSD),

$$\langle \mathbf{r}^2(t)\rangle = \int \mathbf{r}^2 P(\mathbf{r}, t) d^3\mathbf{r} = 6D_1 t\,, \qquad (1)$$

grows linearly in time. The proportionality factor D_1 is called the diffusion constant. The MSD represents an ensemble average in the sense that it measures the spreading of many particles, characterized by the spatial average of $\mathbf{r}^2$ over the probability density function $P(\mathbf{r}, t)$ of finding a particle at position $\mathbf{r}$ at time t. (Angle brackets denote ensemble averages.)

In single-particle analyses such as Nordlund's, by contrast, one measures the trajectory of a single particle in terms of the time series $\mathbf{r}(t')$ over a total measurement time t. Typically one measures a time-averaged MSD

$$\overline{\delta^2(\Delta)} = \frac{1}{t-\Delta}\int_0^{t-\Delta} \big(\mathbf{r}(t'+\Delta) - \mathbf{r}(t')\big)^2 dt'\,, \qquad (2)$$

which integrates the squared displacement between trajectory points separated by the lag time Δ much shorter than t. (Overbars denote time averages.) For Brownian motion of a particle in water at room temperature over long measurement times,

$$\overline{\delta^2} \to 6D_1\Delta\,. \qquad (3)$$

That long-time convergence is essentially identical with the ensemble average in equation 1. The equivalence of time and ensemble averaging is the hallmark

of ergodicity. In that sense, the experiments of Perrin and Nordlund are indeed equivalent.

Anomalous diffusion in living cells

Single-molecule tracking to evaluate time-averaged MSD in cells is usually based on video microscopy of fluorescently labeled molecules (see the box below). Alternatively, one can use indirect tracking with optical tweezers.

What can be seen in such experiments? Ido Golding and Edward Cox at Princeton University have tracked the motion of single messenger RNA molecules in bacteria cells.[2] They found that diffusion of those molecules is anomalous—relative to Brownian diffusion—in two important regards. Parameterizing the time-averaged MSD by

$$\overline{\delta^2} \sim D_\alpha \Delta^\alpha, \tag{4}$$

they found, first of all, that the anomalous diffusion exponent α is about 0.7, which means that the messenger RNA diffusion in vivo has a weaker time dependence than the Brownian diffusion described in equation 3 with $\alpha = 1$. Furthermore, the anomalous diffusion constant D_α deduced from a single trajectory exhibits a pronounced scatter from one trajectory to another (see figure 2a). It looks random.

The randomness and the anomalous time dependence persisted when the Princeton experimenters changed physiological conditions or even disrupted the bacterium's cytoskeletal internal structure. But figure 2b suggests that the confining cell walls play some role in the anomalous results.

It turns out that anomalous diffusion and the irreproducibility of time averages are common in living cells. Similar results have been found for lipid granules in yeast cells,[3] for channel proteins (pore-forming molecules in cell membranes),[4] and for telomeres (chromosomal end parts) in human cell nuclei.[5] Control experiments in artificially dilute environments exhibit anomalous diffusion in which α decreases with increasing concentration of crowding agents and reaches a saturation value at typical physiological conditions.

Those results, in vivo and vitro, challenge our preconceptions. We would anticipate that an unbounded molecule not actively driven by cellular motors exhibits ordinary Brownian motion. Moreover, trained in the spirit of the ergodic theorem, one expects sufficiently long measurements of $\overline{\delta^2}$ to be reproducible.

There's another difference between Brownian motion and diffusion in dense biological environments. For a Brownian process, a measurement of $\overline{\delta^2}$ and therefore D_1 in the time interval $(0, t)$ will be identical to a measurement in the interval $(t, 2t)$. A biological cell, however, is constantly changing and

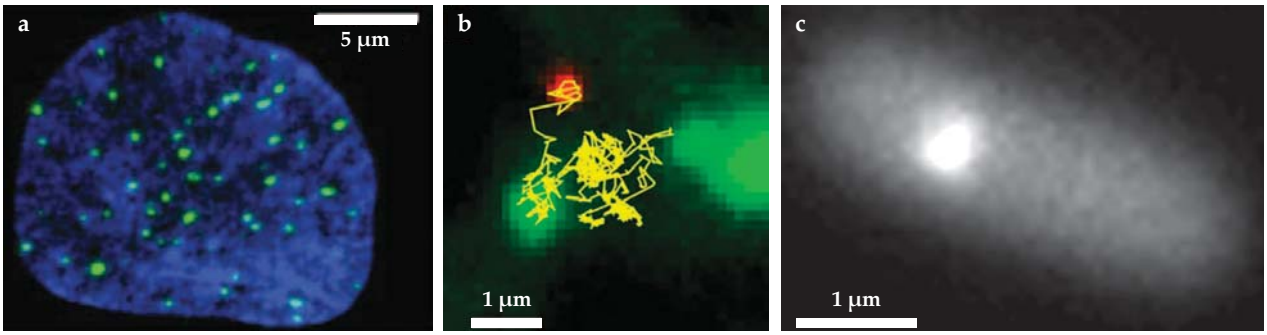
Tracking in vivo

Even in simple cells such as bacteria, the interior is a superdense mix of proteins, nucleic acids, semiflexible polymers such as actin, lipid membranes, and more. To follow individual molecules in such an environment, one has to label them with small fluorescent marker molecules. The panels show such labeled molecules in different living cells: **(a)** chromosome ends (telomeres) in a human cell nucleus,[5] **(b)** trajectory of a channel protein molecule in the plasma membrane of a human kidney cell,[4] and **(c)** a fluorescent messenger RNA tag (bright spot) in an *Escherichia coli* cell (gray oval).[3]

Such markers function as molecular navigation lights. Interacting with an exciting laser field, they fluoresce. For adequate resolution, labeled molecules must be sufficiently far apart and distinguishable from other objects by emission wavelength. A green fluorescent protein (GFP) is ideal in that regard. But an unbound GFP would move too fast to be observed. Beyond the signal-to-noise problem, many fluorescent probes blink and eventually go dark (see the article by Fernando Stefani, Jacob Hoogenboom, and Eli Barkai in PHYSICS TODAY, February 2009, page 34). To overcome those difficulties, experimenters at first tagged only relatively large, slow moving objects.

For a robust signal, one can add many markers to a large single molecule. Multiple marking can, however, change the molecule's behavior.[3] But attaching markers doesn't always compromise the biological system. For telomeres and viruses, fluorescent tagging doesn't interfere with biological activity or dynamics.[4,17] Sufficiently large objects like lipid granules or plastic beads can even be tracked with light microscopes.[2]

Sunney Xie and colleagues at Harvard University have developed a method they call detection by localization, which lets them observe molecules much smaller than messenger RNA. The team's emphasis is on genetic kinetics rather than recording the paths of individual molecules.[14] Advances in both optical technology and the biochemistry of fluorescent markers should usher in a new era in cell biology.



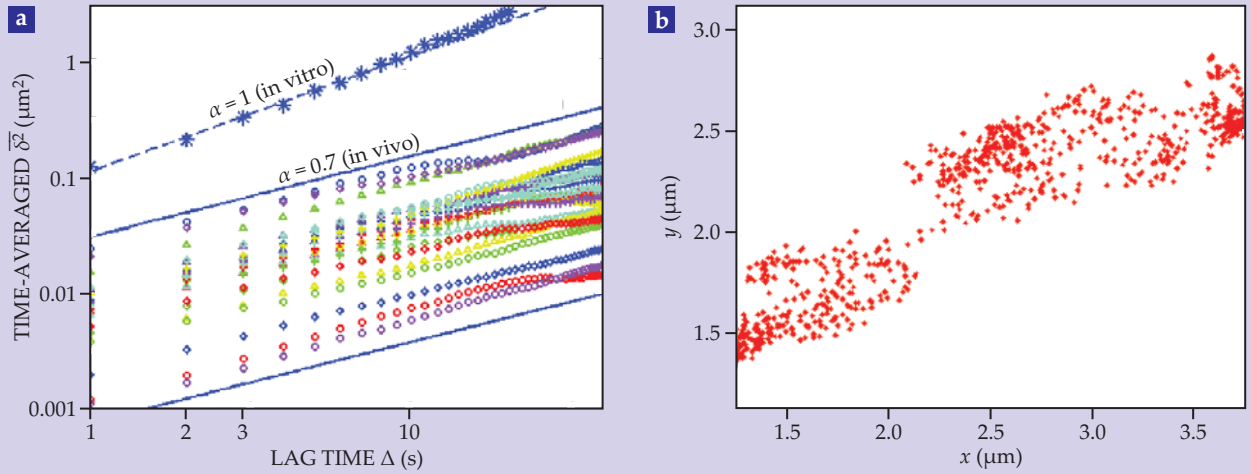


Figure 2. Motion of labeled molecules of messenger RNA in a living *Escherichia coli* bacterium. **(a)** Time-averaged mean squared displacements $\overline{\delta^2}$ of individual trajectories, plotted as functions of lag time Δ in equation 2, display pronounced trajectory-to-trajectory scatter. But all have roughly the same logarithmic slope, corresponding to an anomalous diffusion exponent $\alpha \approx 0.7$ in equation 4. By contrast, the same molecules in water (starred data points) exhibit the $\alpha = 1$ slope of normal Brownian diffusion. **(b)** A single messenger RNA molecule exploring a large fraction of the bacterium's interior collides repeatedly with its confining cell walls. (Adapted from ref. 3.)

aging; some divide and some die. Therefore one might imagine that diffusion properties are not always invariant under time translation.

Models of anomalous diffusion

Let us consider further the origin of anomalous diffusion and its deep connection to ergodic principles.[6] Physicists have been studying anomalous diffusion processes in disordered materials (see the article by Harvey Scher, Michael Shlesinger, and John Bendler in PHYSICS TODAY, January 1991, page 26) and turbulent systems (see the article by Joseph Klafter, Shlesinger, and Gert Zumofen in PHYSICS TODAY, February 1996, page 33). Most of that work involved large ensembles of particles—for example, charge carriers in amorphous semiconductors. Prompted by the new technologies of single-molecule tracking, we now need to deal with single trajectories and consider time averages instead of ensemble averages.

Anomalous diffusion, irreproducibility of time averages, and violation of time-translational invariance are prominent features of a widely applicable stochastic process known as the continuous-time random-walk (CTRW) model. In traditional random-walk models, a particle jumps around a lattice in discrete time steps. In CTRW, by contrast, the particle remains immobile after each jump for a random waiting time τ. One assumes that the distribution of waiting times follows the power-law form

$$\psi(\tau) \sim \tau^{-1-\alpha} \text{ with } 0 < \alpha < 1 . \quad (5)$$

Unlike Einstein's approach to Brownian motion, which corresponds to a finite-average sojourn time between jump events, here the average waiting time diverges. That is, $\langle\tau\rangle = \int_0^\infty \tau\psi(\tau)d\tau = \infty$.

We will see that such scale-free dynamics represents a possible scenario that leads to the strange kinetics under discussion. The CTRW picture can be justified by microscopic models, with α in equation 5 depending on specific system properties. For example, the distribution of waiting times might correspond to a random walker continually caught in potential wells whose depths are distributed exponentially.

In Einstein's theory of Brownian motion, the ensemble-averaged MSD $\langle\mathbf{r}^2(t)\rangle$ grows linearly in time. It's proportional to $t/\langle\tau\rangle$, the number of steps for mean duration $\langle\tau\rangle$. For anomalous diffusion, we use scaling arguments to set $\langle\tau\rangle = \int_0^t \tau\psi(\tau)d\tau \sim t^{1-\alpha}$. That assignment yields the anomalous-diffusion result

$$\langle\mathbf{r}^2(t)\rangle \sim t^\alpha . \quad (6)$$

Thus scale-free waiting times do indeed yield diffusion processes that are slower than Brownian motion.

The CTRW model has a more drastic effect on $\overline{\delta^2}$, the time-averaged MSD. For Brownian motion, time and ensemble averages become identical when the measurement time is long compared to the time scale $\langle\tau\rangle$. But CTRW yields an infinite $\langle\tau\rangle$. No matter how long one measures $\overline{\delta^2}$, it doesn't converge to $\langle\mathbf{r}^2(t)\rangle$. Ergodicity is broken, and $\overline{\delta^2}$ remains random. Averaging $\overline{\delta^2}$ over many individual trajectories, one finds an ensemble average[7,8]

$$\langle\overline{\delta^2}\rangle \sim D_\alpha \frac{\Delta}{t^{1-\alpha}} . \quad (7)$$

Here, unlike in equation 4, the dependence on the lag time Δ is linear, despite the underlying anomalous diffusion. Therefore some care is needed when interpreting experiments; what seems to be normal diffusion may well be anomalous. In equation 7, the anomaly is a kind of aging process. That is, the ensemble average $\langle\overline{\delta^2}\rangle$ decreases with increasing experimental time t.

There's a scaling argument for that aging behavior: For Brownian motion, one has $\langle\overline{\delta^2}\rangle \to 6D_1\Delta = (\langle\mathbf{r}^2(t)\rangle/t)\,\Delta$. One then gets equation 7 by replacing $\langle\mathbf{r}^2(t)\rangle/t$ with $D_\alpha t^{\alpha-1}$.

The CTRW theory describes processes in which the random walker becomes localized for waiting-time periods governed by $\psi(\tau)$. Benoît Mandelbrot proposed a different model of anomalous diffusion, which he called fractional Brownian motion (FBM). Here a stochastic differential equation with random noise $\xi(t)$,

$$\frac{dx(t)}{dt} = \xi(t), \tag{8}$$

describes a component $x(t)$ of $\mathbf{r}(t)$. Unlike CTRW, Mandelbrot's model requires that the dynamics be stationary, which means that the noise correlation function $\langle \xi(t_2)\xi(t_1)\rangle$ depends only on the time difference $|t_2 - t_1|$. In that statistical sense, then, the noise is time-translation invariant. But unlike conventional Brownian noise, the FBM noise is correlated in time. The correlation function goes like $(\alpha - 1)|t_2 - t_1|^{\alpha - 2}$. Its power-law decay with increasing time difference eventually yields anomalous diffusion.

As with CTRW, the FBM ensemble-averaged mean squared displacement increases with time like t^{α}. But FBM's stationary noise restores the equivalence of ensemble and time averages. Indeed ergodicity and stationary dynamics are in many cases related.

Being ergodic and exhibiting no aging, FBM is fundamentally different from CTRW processes. The FBM model can be derived from microscopic scenarios. It might describe, for example, a coordinate of a single particle in an interacting many-body system—a monomer in a polymer chain or some probe particle in a membrane.

Interpreting experiments in living cells

What is the origin of the randomness of time-averaged observables? Is it the nonergodicity of the CTRW model? Or is it a result of spatial inhomogeneities? The latter would imply that the environment sampled by the molecule during its motion through the cell differs from one trajectory to another. Generally, it's hard to determine whether the observed randomness of $\overline{\delta^2}$ is due to ergodicity breaking or random environments.

The specialist community is developing diagnostic tools to answer such questions.[7,9] To distinguish between different stochastic models, one might try to measure the waiting-time distribution $\psi(\tau)$ directly or probe for the aging effects predicted by the CTRW approach. David Weitz's group at Harvard University has measured a long-tailed $\psi(\tau)$ like that of equation 5 for micron-sized beads diffusing in a cross-linked actin network. Recently, Diego Krapf and coworkers at Colorado State University observed power-law waiting times in the motion of channel proteins in membranes of living cells (see figure 3a).[4] They also demonstrated the occurrence of ergodicity breaking and aging by showing that $\overline{\delta^2}$ decreases with increasing measurement time according to equation 7 (figure 3b). All those observed behaviors are predicted by CTRW theory.

But what is the influence of the cell walls that confine molecular motion? While the CTRW model predicts that $\overline{\delta^2}$ is proportional to Δ, the data in figure 3b show power-law scaling proportional to Δ^{α}. As demonstrated theoretically[7,10] and experimentally,[2] confinement induces an apparent scaling of the form $\overline{\delta^2} \sim \Delta^{\beta}$ in the CTRW model, provided that the molecule under observation interacts with the cell boundaries during the experimental time.

A different kind of experiment was performed by one of us (Garini) and coworkers at Bar-Ilan University in Israel.[5] The group recorded the trajectories of individual telomeres within cell nuclei (see the box on page 31) and found pronounced scatter of $\overline{\delta^2}$. Other experiments had also seen such scatter. But the Bar-Ilan team saw something new: The labeled telomeres do not explore the volume of the nucleus. Attached to the large chromosomes, they remain fairly localized.

The observed telomere motion yielded an α of roughly 0.3, close to the $\overline{\delta^2} \sim \Delta^{1/4}$ scaling predicted for motion in a polymer melt in Pierre-Gilles de Gennes's reptation model (see the article by Tom McLeish in PHYSICS TODAY, August 2008, page 40). In that model, a polymer moves like a snake to circumnavigate the topological obstacles created by surrounding polymers in a polymer melt or dense solution. Because of the telomere's connection to the long polymeric chromosome, we expect its diffusion to be governed by FBM. And that's what detailed analysis of the data seems to show. In particular, there's no evidence of aging.

Relevance of anomalous diffusion

Anomalous diffusion of molecules in living cells is slower than normal Brownian processes. Therefore it's sometimes called subdiffusion. What is its biological significance? Might subdiffusion be beneficial for the cell's function? Naively, one might expect Brownian motion to be more efficient because the particles move faster and therefore speed up chemical reactions and the search for physiological targets. Why, then, is anomalous diffusion so common in living systems?

Those questions are difficult to answer with our limited current knowledge of the exact dynamics underlying the various biochemical processes in living cells. Anomalous diffusion of large molecules is related to the high density of the cell environment, which creates many obstacles for the molecule along its path.[11] One can speculate that such crowding is simply a tradeoff between the need to assemble a large number of different molecular and structural components for complex tasks and the requirement that the cell be compact. From that point of view, anomalous diffusion is a consequence of evolutionary optimization.

There are, in fact, several good arguments for why anomalous diffusion might be advantageous. It might, for example, lead to higher reaction efficiency. Biochemical reactions often involve initiation barriers. A reactant that diffuses normally could swiftly escape its target before it's had time to interact.[3] In certain models, the chance of finding a nearby target is explicitly increased by anomalous diffusion.[12]

Recent simulation studies further underline

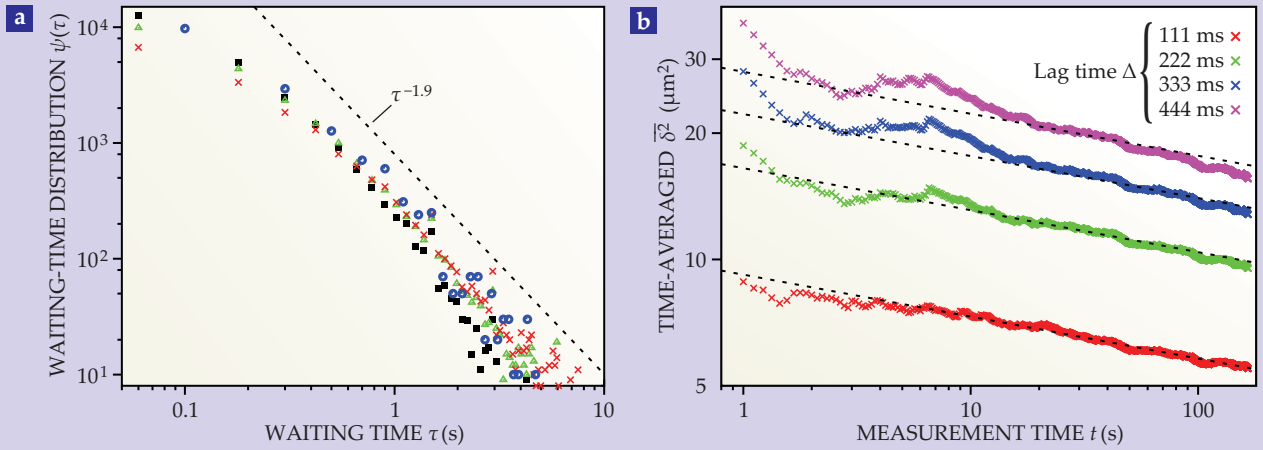


Figure 3. Tracking individual channel protein molecules in human cell walls. **(a)** Observed distribution $\psi(\tau)$ of waiting times τ between observed steps approximates a power-law decay with exponent α of about 0.9 (see equation 5). That's taken as evidence for the continuous-time random-walk (CTRW) model of anomalous diffusion. **(b)** The time-averaged mean squared displacements $\overline{\delta^2}$ for different data-taking lag times Δ (see the color key) all decrease with increasing measurement time t. That's indicative of an aging effect predicted by the CTRW model (see equation 7). (Adapted from ref. 4.)

the biological significance of anomalous diffusion.[13] Enzymatic reaction cascades have been reported in which subdiffusion optimizes the final product by keeping intermediate products from wandering off. Also, it's been shown that some cellular defense mechanisms with very low binding rates to their targets are rendered surprisingly efficient by subdiffusion.

Another important idea relates subdiffusion to the organization of the cell nucleus.[5] Most of the DNA in human cell nuclei is tightly wound in 46 chromosomes. The chromosomes are spatially separated into territories. In the box on page 31, each of the fluorescent molecules in panel a is presumably ensconced in such a territory.

That separation of chromosomes is essential for the cell's genomic function. It may be that such ordering into territories is achieved by physical barriers. Alternatively, the territorial separation might be connected to the extremely slow $\Delta^{1/4}$ diffusion measured for the telomeres, which may simply be due to the crowded and viscous environment. In that case, the chromosomes remain compartmentalized without the need for physical boundaries; they are like tightly packed commuters in a subway car at rush hour, where jamming maintains the ordered state.

Thus far, the tracking and simulation results are just single pieces of the puzzle. But they already show that subdiffusion and efficient cellular dynamics are not mutually exclusive. Recent bioinformatics findings suggest that critically interacting parts of the genome are often arrayed in close proximity on the DNA. That arrangement provides another argument for the benefits of anomalous diffusion. Efficient cell function requires reactants to be produced near their intended reaction centers. Anomalous diffusion can ensure efficiency by keeping reactants from escaping.

Such a local picture of cellular regulation and signaling would not only be compatible with anomalous diffusion, it would also be energetically economical and make possible high physiological accuracy with low copy numbers of individual reactants. Location-specific single-molecule targeting could thus become the new paradigm for cell biology, replacing the conventional conception of the cell as a small, well-mixed reaction flask. It would seem that cells have learned ways to use subdiffusion to their advantage.

Nonetheless, some processes involving transfer of chemical information or cargo have to be fast. In such cases, anomalous diffusion poses problems. When necessary, cells might overcome such problems by active motion along cytoskeletal motorways, along which motor proteins move cargo. Inside some long human neurons, for instance, small vesicles are transported along tubular structures for up to a meter. Such motion is "super-diffusive" in the sense that the exponent α in equation 4 exceeds 1.

Michael Elbaum and coworkers at Israel's Weizmann Institute of Science have investigated such behavior by tracking microspheres in living cells. Like the groups that track molecules, they also find that the time-averaged MSD is random from one trajectory to another.

Trends

While the experiments we have surveyed here focus mainly on the diffusion of single molecules in living cells, the single-molecule approach is far more general. Recent experiments show how a cell's fate can be determined by a stochastic single-molecule switch. It's known that genetically identical cells can come in different phenotypes. For example, *Escherichia coli* bacteria with the same genotype can have different resistivities to antibiotics. Sunney Xie's group at Harvard University has used single-molecule techniques to reveal the mechanism leading

to the creation of such a phenotype.[14] Interestingly, they find that whether the cell develops into one phenotype or another depends on a single binding event of a repressor molecule to the DNA.

Since the cell's fate in such a scenario is determined by a single molecular event, one is again far from the realm of conventional thermodynamics, where the phase of a macroscopic system never turns on a single microscopic event—*pace* Schrödinger's cat. Finding a case where the flipping of one molecular coin actually does determine the fate of a living organism has been made possible by single-molecule detection techniques.

As investigators in this young field accumulate more and better data, they will have the opportunity to categorize the motions and reactions of a wide variety of molecules in living cells and relate them to cellular functions. For example, we would like to see the correlation between the exponent α and the size of diffusing molecules. When do smaller molecules, usually unhampered by dense cellular environments, exhibit normal ergodic diffusion?

Future optical challenges include improving temporal resolution and finding smaller and brighter light emitters that don't disturb biological function. Finally, the fundamental difference between ensemble and time averages is certainly not limited to a single observable like the mean squared displacement of a particle diffusing in living cells. Such departures from ergodicity have broad consequences for the dynamics of disordered inanimate systems, in which single-particle behavior can be very different from that of the ensemble.

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